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I need the following papers for examinatin of SN 09/218,481

1. Plate et al, 1992, Nature, 359:845-848

2. Kim et al, 1993, Nature, 362:841-844.

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Thanks
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gelatinase A was incubated with β-AP_{10-20}, the peptide was effectively hydrolysed into two fragments (Fig. 3, I and II). These proteolytic fragments correspond to β-AP_{10-16} (Tyr-Glu-Val-His-His-Gln-Lys) and β-AP_{17-20} (Leu-Val-Phe-Phe), indicating that gelatinase A hydrolyses the Lys 16-Leu 17 bond, which accords with the substrate specificity of APP secretase (Fig. 2*d*). The isolated 41K enzyme hydrolyses both β-AP_{10-20} and gelatin, whereas the 57K enzyme hydrolyses only gelatin (data not shown).

The 100K APP effectively inhibits β-AP_{10-20} hydrolysis of activated gelatinase A, although ^{3}H-gelatin hydrolysis is inhibited far less (Fig. 3, III, and Fig. 4*a*). The APP concentration giving 50 per cent inhibition (IC_{50}) was ~90 nM for β-AP_{10-20} hydrolysis and ~1.5 μM for gelatinolysis. Kinetic analysis at various concentrations of β-AP_{10-20} and inhibitor indicated that the K_m of gelatinase A for β-AP_{10-20} is ~130 μM, and that 100K APP inhibits β-AP_{10-20} hydrolysis in a competitive manner (Fig. 4*b*). From the data shown in Fig. 4*a* and *b*, the inhibition constant (K_i) of 100K APP for β-AP_{10-20} hydrolysis is estimated as ~40 nM.

Gelatinase A exists in the plasma membrane of cells in lines[16] and its proenzyme is activated to the 57K and 41K forms by a plasma membrane fraction[17]. We have detected both gelatinase A and soluble APP in high amounts in conditioned media of human cancer cell lines such as glioblastoma, neuroblastoma and EJ-1 (Fig. 1*a*), and in human cerebrospinal fluids. Overexpression of the APP gene in neuronally differentiated mouse embryonal carcinoma cells is known to cause aberrant processing of APP and degenerative death of these cells[18]; also, both the secretory APP and β-AP forms are produced in culture and in normal individuals[19-21]. Our results suggest two possible functions for gelatinase A: it may produce secretory APP on the plasma membrane and it may degrade soluble β-AP in the extracellular matrix. Both actions would prevent β-AP accumulation in the extracellular matrix. When APP is overproduced under certain conditions, however, it may inhibit these activities of gelatinase A, favouring the extracellular deposition of β-AP. □

Received 30 November 1992; accepted 18 February 1993.

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ACKNOWLEDGEMENTS. We thank H. Yasumitsu, Y. Suzuki, N. Koshikawa and Y. Nomizu for technical support and for discussion. This work was supported in part by a grant from the Oriental Yeast Co. (Tokyo) and the Special Coordination Funds of the Science and Technology Agency of Japan.

Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*

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THE development of new blood vessels (angiogenesis) is required for many physiological processes including embryogenesis, wound healing and corpus luteum formation[1,2]. Blood vessel neoformation is also important in the pathogenesis of many disorders[1-5], particularly rapid growth and metastasis of solid tumours[3-5]. There are several potential mediators of tumour angiogenesis, including basic and acidic fibroblast growth factors, tumour necrosis factor-α and transforming factors-α and -β[1,2]. But it is unclear whether any of these agents actually mediates angiogenesis and tumour growth *in vivo*. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumour cells and expressed in human tumours *in situ*. To test whether VEGF may be a tumour angiogenesis factor *in vivo*, we injected human rhabdomyosarcoma, glioblastoma multiforme or leiomyosarcoma cell lines into nude mice. We report here that treatment with a monoclonal antibody specific for VEGF inhibited the growth of the tumours, but had no effect on the growth rate of the tumour cells *in vitro*. The density of vessels was decreased in the antibody-treated tumours. These findings demonstrate that inhibition of the action of an angiogenic factor spontaneously produced by tumour cells may suppress tumour growth *in vivo*.

VEGF has several attractive features as a mediator of normal and pathological angiogenesisis. VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer *in vivo*[6-9]. Its high-affinity binding sites are localized only on endothelial cells in tissue sections[10]. VEGF was also purified independently as a tumour-derived vascular permeability factor[11,12]. By alternative splicing of messenger RNA, VEGF may exist in four different homodimeric molecular species, each monomer having 121, 165, 189 or 206 amino acids, respectively ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$)[13-15]. $VEGF_{121}$ and $VEGF_{165}$ are soluble proteins, whereas $VEGF_{189}$ and $VEGF_{206}$ are bound to heparin-containing proteoglycans in the cell surface or in the basement membrane[16]. A temporal and spatial correlation exists between VEGF mRNA expression and physiological proliferation of blood vessels in the ovarian corpus luteum[17] or in the developing brain[18]. Several observations suggest that VEGF may be potentially involved in tumour angiogenesis. A variety of transformed cell lines express the VEGF mRNA and secrete VEGF[19,20]. Also, *in situ* hybridization studies demonstrate expression of VEGF mRNA at high level in various human tumours, including the highly vascularized glioblastoma multiforme and capillary haemangioglastoma[21-24]. Furthermore, expression of $VEGF_{165}$ or $VEGF_{121}$ confers on Chinese hamster ovary cells the ability to form tumours in nude mice[25].

Proof of the role of VEGF in tumour angiogenesis require the demonstration that inhibition of VEGF action preven tumour growth *in vivo*. The availability of specific monoclo antibodies capable of blocking VEGF-induced angiogenesi *vivo* and *in vitro*[26] allowed us to test the hypothesis dire The human A673 rhabdomyosarcoma, G55 glioblastoma m forme and SK-LMS-1 leiomyosarcoma cell lines are tumori in nude mice, express the VEGF mRNA (ref. 20 and unpub

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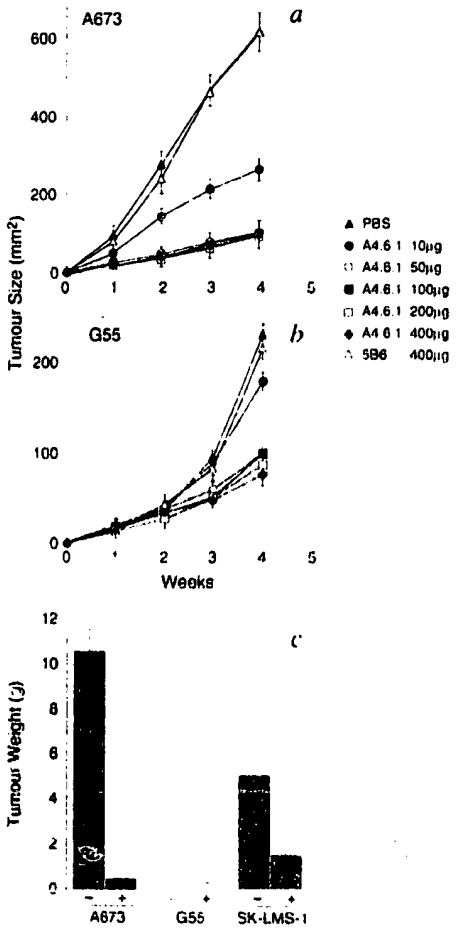

FIG. 1 Effects of anti-VEGF antibody on tumour size (*a*, *b*) and weight (*c*). A673 (CRL 1598) and SK-LMS-1 (HTB 88) cell lines were from the ATCC. A series of cell lines were established from glioblastoma multiforme specimens[30]. The G55 cell line was reproducibly tumorigenic and secreted VEGF. Cells were cultured in the presence of DMEM/F12 supplemented with 10% FBS, 2 mM glutamine and antibiotics and passaged weekly at a split ratio of 1:20. For injection into nude mice, cells (passages 3–5) were dissociated from stock plates in the presence of 125 mM NaCl, 5 mM KCl, 50 mM HEPES, 5 mM glucose, 1 mM EDTA, pH 7.4, washed once and then resuspended in PBS at the desired density. Female Beige nude/xid mice (6–10 week old) (Charles River, Wilmington, DE) were injected subcutaneously with 1×10^6 cells in the dorsal area in a volume of 0.1 ml. The neutralizing anti-VEGF antibody A.4.6.1 (ref. 26) was injected intraperitoneally in a volume of 0.2 ml twice weekly at various doses. As controls, an antibody of the same isotype (IgG_1), directed against the gp120 protein[31] (antibody 586) or PBS were used. Each group comprised 10 mice. In *a*, the antibody treatment was initiated immediately after cell injection. In *b*, it was initiated after 7 days. At the end of the experiment, animals were killed by CO_2 inhalation. Tumour sizes were determined by multiplying the width by the length. Values shown are means ± s.e.m. Statistical analysis was done by a two-sided unpaired *t*-test following a one-way analysis of variance. Comparison of tumour size of the PBS group with those of animals treated with antibody A.4.6.1 at doses at 50 μg or higher revealed *P* values <0.0001 for both types of tumour. *P* values for the 10 μg dose were 0.003 for the A673 and 0.011 for the G55. Tumour sizes in the group treated with antibody 586 were not different from those injected with PBS alone. *c*, Weights of tumours derived from A673, G55 or SK-LMS-1 cells. Data shown reflect the response to 100 μg twice weekly of antibody A.4.6.1. Values are means ± s.e.m. Plus and minus signs denote the presence or absence of antibody treatment. A673 and G55 tumours were collected 4 weeks after tumour cell injection. Animals bearing tumours derived from SK-LMS-1 cells were killed after 10 weeks.

observations), and release VEGF in the culture medium (Table 1). The A673 cells secrete the highest amounts of VEGF, the SK-LMS-1 cells the lowest. Such cells may thus provide a model to test the hypothesis that VEGF is a paracrine mediator of tumour growth in an *in vivo* system.

We injected the tumour cells into nude mice. Animals then received various doses of an anti-VEGF monoclonal antibody, a control antibody or PBS twice weekly. Figures 1*a* and *b* illustrate the growth, as a function of time, of tumours derived from A673 or G55 cells. Although the control antibody had no appreciable effect, the anti-VEGF antibody was growth inhibitory to both types of tumour. But the magnitude of the response was greater in the rhabdomyosarcoma, the more rapidly proliferating and therefore the more angiogenesis-dependent tumour. As little as 10 μg twice weekly of the antibody resulted in a significant inhibition. In both tumours, a maximal effect was achieved with antibody doses of 50–100 μg. Growth inhibition was achieved not only when the anti-VEGF antibody administration was started immediately after tumour cell inoculation but also when such treatment was initiated a week later, when the

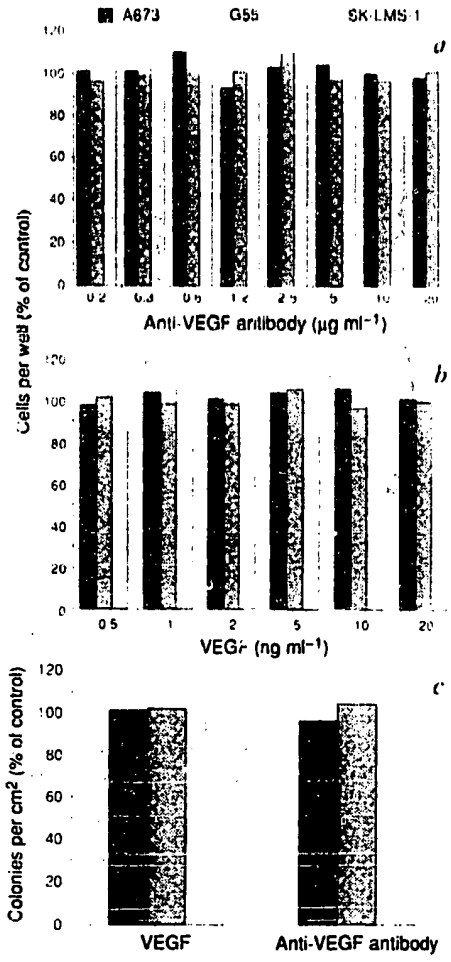

FIG. 2 Effects of VEGF or anti-VEGF antibody on the growth of tumour cell lines in plastic or in soft agar. For proliferation assays, tumour cells were seeded at the density of 7×10^3 per well in 12 multiwell plates in the presence of DMEM/F12 supplemented with 10% FBS, 2 mM glutamine and antibiotics: $rhVEGF_{165}$ or antibody A.4.6.1 were added at the indicated concentrations. After 5 days, triplicate wells were exposed to trypsin and cells were counted in a Coulter counter. Soft agar colony formation assay was done as described[29]. $rhVEGF_{165}$ was added at the final concentration of 20 ng ml^{-1}; antibody A.4.6.1 was tested at the final concentration of 1 μg ml^{-1}.

tumours were already established (Fig. 1*b*). The SK-LMS-1 cells had the slowest growth rate and tumours could not be measured for several weeks. The anti-VEGF antibody also inhibited their growth. Figure 1*c* shows the weight of tumours derived from the three cell lines after treatment with 100 μg twice weekly of anti-VEGF antibody in a representative experiment. The decreases in tumour weight were 96% for the A673, 80% for the G55 and 70% for the SK-LMS-1 cells. Such profound effects are consistent with inhibition of a fundamental mechanism of tumour growth. The lack of complete inhibition may be explained by the presence of additional angiogenic factors. For example, basic or acidic fibroblast growth factors are produced by glioma[27] and sarcoma[28] cell lines.

To verify that such *in vivo* effects do not result from inhibition of autocrine actions of VEGF or from direct cytotoxicity of the antibody, we tested the anti-VEGF antibody or VEGF (Fig. 2) for their ability to affect the *in vitro* growth of tumour cell lines, both in plastic[25] and in soft agar[29]. Neither the antibody nor VEGF had any effect on the growth rate or colony formation in any of the cell lines tested. These findings agree with previous studies that showed VEGF to be an endothelial cell-specific mitogen[6].

Microscopic examination of tumours and other tissues from animals injected with A673 or G55 cells did not reveal marked differences between control and anti-VEGF antibody-treated groups in several histological parameters. Multifocal coalescing foci of necrosis interspersed among masses of tumour cells were present in both rhabdomyosarcoma and glioblastoma multiforme sections. Metastases to lungs or liver were very rare. Inflammatory cell infiltrates were minimal and oedema was virtually absent in all tumour sections examined. But the density of vascular elements was decreased in the anti-VEGF antibody-treated tumours when compared with controls. This was confirmed by immunocytochemistry with an antiserum directed against factor VIII-related antigen (Fig. 3*a–c*). This decrease in vascular density was more prominent in sections from the rhabdomyosarcoma than from the glioblastoma. This is consistent with the more dramatic response of the rhabdomyosarcoma to the anti-VEGF antibody.

TABLE 1 Concentrations of VEGF by ELISA in the conditioned medium of tumour cell lines

	48 h	96 h	144 h
A673	5	45	85
G55	2	18	41
SK-LMS-1	<0.2	2	1[a]

Cells were cultured in 10-cm tissue culture dishes in the presence of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Subconfluent cultures were washed and then incubated in serum-free medium. At the indicated time points, aliquots of medium were collected and subjected to enzyme-linked immunosorbent assay (ELISA). The VEGF ELISA was done as described[16]. Values are in ng ml^{-1}. The limit of sensitivity of the assay was 0.2 ng ml^{-1}.

To elucidate the expression pattern of VEGF mRNA, we did *in situ* hybridization with a probe specific for VEGF on tumours derived from A673 or G55 cells. We found similar patterns in the two types of tumour (Fig. 3*d–g*). In tumours from untreated animals, VEGF expression was not uniform but was at the highest level in clusters of cells at the periphery of cell cords surrounded by necrotic areas. Numerous blood vessels were identified within these tumour cell cords (Fig. 3*a*, *d*, *f*). Remarkably, this pattern is very similar to that recently described in glioblastoma multiforme specimens *in situ*[22,23]. This is consistent with the hypothesis that hypoxaemia triggers expression and release of VEGF in focal areas of the tumour[22,23]. This would generate a spatial gradient of endothelial cell proliferation and angiogenesis toward ischaemic areas, permitting cell proliferation and tumour expansion. In the anti-VEGF antibody-treated groups, VEGF expression was also highest in tumour cells facing necrotic areas. But blood vessel lumina were rarely observed (Fig. 3*b*, *e*, *g*). These findings strongly support the hypothesis that inhibition of angiogenesis is the event limiting tumour growth in the anti-VEGF antibody-treated animals.

The probe that we used does not discriminate between the

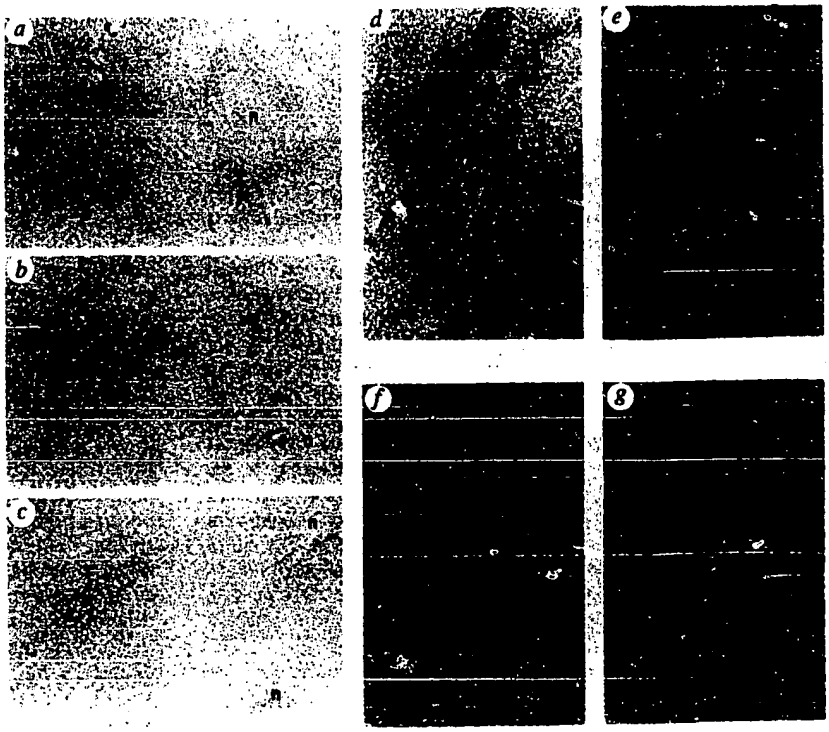

FIG. 3 Immunocytochemical stain for factor VIII-related antigen (*a–c*) and *in situ* hybridization with a probe specific for VEGF (*d–g*). *a*, *c*, A673 tumours from untreated or *b*, antibody-treated animals. In *c*, normal rabbit serum was added instead of anti-factor VIII antiserum. Tissues were fixed in the presence of 10% neutral buffered phosphate formalin and then paraffin-embedded. Immunocytochemistry was done with the avidin-biotin-peroxidase kit with diaminobenzidine as the chromogen, according to the instructions of the manufacturer (Vector Labs, Burlingame, CA). The primary antiserum (rabbit anti-human factor VIII: Dako, Carpenteria, CA) or normal rabbit serum was added at the dilution of 1:100 for 30 min at room temperature. *d*, *e*, Brightfield images of A673 or *f*, *g*, G55 tumour sections hybridized with an antisense RNA probe specific for human VEGF. *d*, *f*, Untreated. *e*, *g*, Treated with anti-VEGF antibody. Individual arrows point to blood vessel lumina. Double arrows point to VEGF-expressing cells. *In situ* hybridization was done on unfixed frozen sections using a 1 kilobase probe representing residues 924–1,920 of a human VEGF complementary DNA, as described[32]. No appreciable hybridization was observed in sections incubated with the sense probe. Tumours used for immunocytochemistry or *in situ* hybridization were collected 4 weeks after cell inoculation. The dose of anti-VEGF antibody was 100 μg twice weekly. Untreated animals received PBS alone. n, Necrotic areas. Scale bar, 100 μm.

various molecular species of VEGF. But most of the endothelial cell mitogenic activity released by the tumour cells *in vitro* was strongly retained by heparin-sepharose and was eluted in the presence of 0.9 M NaCl (unpublished observations). This chromatographic behaviour is consistent with $VEGF_{165}$ but not with the other isoforms of VEGF[16]. This molecular species of VEGF is soluble after secretion and therefore is largely free to diffuse and reach its receptors in the vasculature[6,16].

Our findings demonstrate for the first time, to our knowledge, that blocking the action of a paracrine mediator that acts on the vasculature may have a significant or even dramatic inhibitory effect on tumour growth and emphasize the significance of VEGF as an important mediator of tumour angiogenesis. Therefore, blocking VEGF action has the potential to be of therapeutic significance for several highly vascularized and aggressive malignancies. □

Received 21 January; accepted 8 March 1993

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ACKNOWLEDGEMENTS. We thank M. Westphal and W. Hamel for the generous gift of the G55 cell line; R. Shillaby for his help and advice with nude mice experiments; W. Young and J. Silva for excellent animal work; D. Finkle for histology; C. Kyle for immunocytochemistry and N. Singh for ELISA; D. Gallman and E. Gilkerson for statistical analysis; J. Baker, G. Vehar, M. Cronin and R. Thomas for reading the manuscript and B. Fendly and E. Patzer for helpful discussions and advice.

Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*

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THE prototype members of the heparin-binding fibroblast growth factor (FGF) family[1–6], acidic FGF (FGF-1) and basic FGF (FGF-2), are among the growth factors that act directly on vascular cells to induce endothelial cell growth and angiogenesis. *In vivo*, the role of the FGF prototypes in vascular pathology has been difficult to determine. We report here the introduction, by direct gene transfer into porcine arteries, of a eukaryotic expression vector encoding a secreted form of FGF-1. This somatic transgenic model defines gene function in the arterial wall *in vivo*. FGF-1 expression induced intimal thickening in porcine arteries 21 days after gene transfer, in contrast to control arteries transduced with an *Escherichia coli* β-galactosidase gene. Where there was substantial intimal hyperplasia, neocapillary formation was detected in the expanded intima. These findings suggest that FGF-1 induces intimal hyperplasia in the arterial wall *in vivo* and, through its ability to stimulate angiogenesis in the neointima, FGF-1 could stimulate neovascularization of atherosclerotic plaques. Potentially, gene transfer of FGF-1 could also be used as a genetic intervention to improve blood flow to ischaemic tissues in selected clinical settings.

The FGF prototypes lack a classic signal sequence[1] (ss) for secretion, making it difficult to study their biological effects as extracellular polypeptides. It is now possible, however, to deliver recombinant genes directly into vascular cells at specific sites *in vivo*[7–12] to determine their effects in the arterial wall. A secreted form of the FGF-1 gene was derived by ligation of the signal sequence from the hst/KS3 (FGF-4) gene to the 5′ end of the open reading frame of FGF-1 (ref. 13) in the pMEX neo eukaryotic expression vector[14]. The pMEX neo-ss-hsk/KS3:FGF-1 expression vector plasmid was transfected into porcine iliofemoral arteries by direct gene transfer[8,15,16], and controls were transfected with the *E. coli* β-galactosidase gene. The presence of the ss-hst/KS:FGF-1 plasmid was confirmed using polymerase chain reaction (PCR) in transfected iliofemoral arterial segments (Fig. 1*a*, lanes 1 and 2) but not in nontransfected carotid artery segments from the same pig (data not shown), and the presence of its messenger RNA was confirmed by reverse transcription PCR (Fig. 1*b*, lane 4). Expression of recombinant FGF-1 protein was confirmed by immunohistochemistry in transduced arterial segments. Porcine arteries transfected with ss-hst/KS:FGF-1 had immunoreactive protein primarily in the intima, including the endothelium, 21 days after transfection, whereas no FGF-1 protein was detected in arteries transduced with the β-galactosidase expression vector (Fig. 2*a*–*c*).

To evaluate the response of the arterial wall to expression of ss-hst/KS:FGF-1, the transfected artery segments were examined by light microscopy 21 days after gene transfer. Animals transduced with β-galactosidase showed minimal intimal thickening in iliofemoral artery segments, in contrast to the ss-hst/KS:FGF-1-transduced arteries (compare Fig. 3*a* and *b*). By quantitative morphometry, the intimal to medial ratio was more than sixfold greater in FGF-1 than β-galactosidase-transduced vessels (0.27 ± 0.06 versus 0.04 ± 0.01, $P = 0.003$). Finally, in several experimental subjects, expression of ss-hst/KS:FGF-1 induced the formation of capillaries in the neointima (Fig. 4*a*, *b*), an effect not observed with the control.

Thus expression of secreted recombinant FGF-1 induced significant intimal proliferation and angiogenesis *in vivo*. Angiogenic factors[1,2,6] have been classified previously into two categories: those that act directly on vascular endothelial cells to stimulate locomotion and mitosis and those that act indirectly to induce host cells to release growth factors that target the endothelial cell. In addition, because the FGF prototypes lack a classic signal sequence for secretion, their normal mode of release is not fully understood. They are detected after arterial